Analytical HPLC Protocol

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I. Sample Preparation
   a. Dissolve a small amount of sample in H2O or H2O/ACN
   b. Evaporate any halogenated solvents!
   c. Filter samples through a 0.2/0.22 μm filter into an Agilent HPLC vial. The sample needs to be at least 0.5 mL in volume unless using an insert.
   d. Place into autosampler.

II. HPLC Setup
   a. Make sure all bottles have > 400 mL of solvent before starting. The computer doesn't like if the solvent level dips below 300 mL.
   b. Lines:
      i. A1 – 5% ACN, 95% H2O, 0.1% TFA
      ii. A2 – H2O
      iii. B1 – 100% ACN, 0.1% TFA
      iv. B2 – ACN
   c. All solvents should be made of HPLC grade solvents (4L amber bottles) and filtered through a 0.2/0.22 μm filters.

III. Method Setup
   a. Method→New Method
   b. Method→Edit Entire Method
   c. Follow the windows. Most things are fine on the default settings, so only change the following:
      i. "Set up pump" window
         1. Flow → 0 mL/min (this will be the flow rate after the method is done. To stop the pumps, this should be 0).
         2. Stop time → length of run (this is the stop time for the UV detection and pumps)
         3. Solvents → using A1/B1 or A2/B2 pair?
         4. Timetable → when creating a timetable, start with high water content and replace it with ACN such that at the end of the run, you flush it with 100% ACN. A sample timetable is shown below. Remember that A1 contains 5% ACN, so 0% B is actually 5% ACN. The instrument always makes gradients, so if you want the %B to be constant, add both the start and the stop time (#5,6):

	Time	%B	Flow	Max Pressure
1	0	0	1	Leave blank--
2	10	5	1	Default is 400 mbar
3	15	25	1	
4	20	50	1	
5	25	100	1	
6	29	100	1	

7	30	0	1	
8	35	0	1	

ii. "Set up injector" window
    1. Use 'standard injection' in general. For 'injection with needle wash', add another vial to autosampler containing filtered ACN. Only need this when working with something that is nasty or potentially aggregates/self assembles.
    2. Change injection volume to anything between 0-100 μL.
iii. "DAD signal" window
    1. Click on all the wavelengths you want the computer to monitor and record (up to 4)
    2. For cyclic peptides without aromatic residues, use 210, 230 nm; for ones with aromatics, also use 280.
iv. "Instrument Curves" window
    1. Select all signals you want to see during the run-generally %A, %B, wavelengths

d. Save Method

IV. Starting Up and Equilibrating Column
a. Turn on all components of the HPLC instrument (should be on already) and the icons on the software (Instrument 1 Online) and wait until everything is green on the screen.
b. Open the purge valve (black valve, 3rd block of the HPLC). This sends the solvents to waste directly.
c. *To manually control the pumps, left click on the pump icon→set up pump and change "Flow" and hit OK. To turn off pump at any time, put Flow=0. Ignore the timetable on the bottom.
d. Run 100% A1, followed by 100% B1 (if using A1/B1 combo) at 5 mL/min for 1.5 min each.
    i. Left click on the pump icon→Set Up Pump
    ii. Flow→5
    iii. Stop Time→1.5
    iv. Make sure the A1/B1 circles are highlighted
    v. Put in 0% B
    vi. Click OK. This should shut off the pump after 1.5 min, but it doesn't sometime, so don't leave the instrument.
    vii. Repeat for 100% B.
    viii. Close the purge valve
e. Wash the column with B1 for 5 min at max flow rate of column (1 ml/min)
f. Wash the column with A1 for 5 min at max flow rate of column (1 ml/min)
g. Turn off pumps by setting "Flow"=0

V. Setting Up a Run

a. Load method and check to make sure it is correct. Save any changes made at this point or the computer will forget them.
b. Change sequence parameters
    i. Left click on autosampler picture→Sequence Parameters
    ii. Change operator name
    iii. Change subdirectory (add new subdirectory by putting in a name and clicking ok on the popup window)
    iv. Data file should be C:\Chem32\1\DATA\ and the numbering on Auto
c. Set up sequence
    i. Left click on autosampler picture→Sequence Table
    ii. Put in the vial #, sample name
    iii. Select your method – Important! This is what the computer will run, no matter what you have open on the screen
    iv. Inj/vial (leave on 1 unless you want multiple runs sequentially from the same vial)
    v. Leave everything else as is ('Sample'-sample, everything else blank)
d. Check solvents
    i. Left click on solvent bottles→Solvent Bottle Filling
    ii. Check to make sure the numbers match the amount of solvents actually in the bottles.
e. Click Start to start the run.
f. Double click on the window or on the three-way arrows near the center of the screen that tracks the UV absorbance to bring up a larger window to monitor run. There is a couple second delay between the UV reading and when the sample comes out. Use the arrows to change timescale. The solvent front will generally come out in the first 1.5 min.

VI. Clean Up
a. Set up a new run with "CLEANING.M" method. It should run pure ACN (B2) and H2O (A1) through the column for about 4 min each, then 50% of each for 3 min. The column is stored in 50% ACN/H2O. Either inject filtered ACN or set injection volume to 0.
b. After run is finished, open purge valve and flush pumps for 1.5 min with 100% ACN then 100% H2O at 5 mL/min.
c. Close purge valve.
d. Leave the instrument turned on, but turn off pumps on software. Only turn off the DAD if no one wants to use the instrument that week.

VII. Data Analysis
a. To view data, open Instrument 1 Offline program
b. Open your subdirectory folder on the left side of the screen under the data analysis tab and choose the bottom file for the latest run.
c. Play around with the software until you have desired graph…